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Article (Published Version)

Lisco, Andrea, Vanpouille, Christophe, Tchesnokov, Egor P, Grivel, Jean-Charles, Biancotto, Angélique, Brichacek, Beda, Elliott, Julie, Fromentin, Emilie, Shattock, Robin, Anton, Peter, Gorelick, Robert, Balzarini, Jan, McGuigan, Christopher, Derudas, Marco, Götte, Matthias et al. (2008) Acyclovir is activated into a HIV-1 reverse transcriptase inhibitor in herpesvirus-infected human tissues. *Cell Host and Microbe*, 4 (3). pp. 260-270. ISSN 1931-3128

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Acyclovir Is Activated into a HIV-1 Reverse Transcriptase Inhibitor in Herpesvirus-Infected Human Tissues

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DOI 10.1016/j.chom.2008.07.008

SUMMARY

For most viruses, there is a need for antimicrobials that target unique viral molecular properties. Acyclovir (ACV) is one such drug. It is activated into a human herpesvirus (HHV) DNA polymerase inhibitor exclusively by HHV kinases and, thus, does not suppress other viruses. Here, we show that ACV suppresses HIV-1 in HHV-coinfected human tissues, but not in HHV-free tissue or cell cultures. However, addition of HHV-6-infected cells renders these cultures sensitive to anti-HIV ACV activity. We hypothesized that such HIV suppression requires ACV phosphorylation by HHV kinases. Indeed, an ACV monophosphorylated prodrug bypasses the HHV requirement for HIV suppression. Furthermore, phosphorylated ACV directly inhibits HIV-1 reverse transcriptase (RT), terminating DNA chain elongation, and can trap RT at the termination site. These data suggest that ACV anti-HIV-1 activity may contribute to the response of HIV/HHV-coinfected patients to ACV treatment and could guide strategies for the development of new HIV-1 RT inhibitors.

INTRODUCTION

HIV-1 infection is usually accompanied by infection with other microbes, HIV-1 copathogens, which either pre-exist in the human body or invade it de novo. The replication of HIV-1 copathogens is frequently promoted in HIV-1-infected hosts, and their suppression is often beneficial for the clinical course of HIV disease (Corey, 2007; Jacobson and Mills, 1988). Human herpesvirus (HHV) infections are commonly associated with HIV-1. In particular, herpes simplex virus 2 (HSV-2) infection is associated

with an increase in HIV-1 transmission (Cohen, 2004; Freeman et al., 2006) and worsens the clinical course of HIV disease. HSV-2 reactivation may lead to increased plasma HIV-1 levels, thereby adversely affecting survival rates (Corey et al., 2004; Schacker et al., 2002).

Acyclovir (ACV) is a guanosine nucleoside analog particularly active against HSV-2 as well as against the other α -HHVs (HSV-1 and VZV) (Elion, 1983). It also inhibits, although with lower potency, the replication of the β -HHVs (CMV, HHV-6, and HHV-7) and of the γ -HHVs (EBV and HHV-8) (De Clercq et al., 2001).

The mechanism of HHV suppression by ACV is well understood. ACV is phosphorylated in HHV-infected cells by viral-encoded kinases. The resulting monophosphate derivative (ACV-MP) is then converted into ACV triphosphate (ACV-TP) by cellular enzymes and is subsequently incorporated by the viral DNA polymerase into the nascent viral DNA chain, causing its obligate termination (Reardon and Spector, 1989). Generally, the sensitivity of different HHVs to ACV is determined by the extent of its phosphorylation by HHV kinase and by the rate of incorporation of ACV-TP into the viral DNA chain (De Clercq et al., 2001). The mechanism described above explains why ACV does not directly affect viruses other than HHVs, including HIV-1. Nevertheless, early clinical trials indicated that ACV treatment may result in survival benefits in cohorts of HIV-1-infected patients with a high incidence of clinically identifiable HHV (Ioannidis et al., 1998). In agreement with these data, recent trials showed that, in HSV-2-coinfected individuals, the suppression of HSV-2 by ACV is accompanied by reduction of the plasmatic, genital, and rectal HIV-1 load (Baeten et al., 2007; Delany et al., 2007; Dunne et al., 2008; Nagot et al., 2007; Zuckerman et al., 2007). This decrease was attributed to the suppression of HSV-2-mediated inflammation indirectly reducing HIV-1 load.

Here, we report on the direct activity of ACV on HIV-1 and, specifically, on the inhibition of HIV-1 reverse transcriptase

(RT) by ACV-TP. We demonstrate that, consequently, ACV is an anti-HIV-1 agent in human lymphoid, rectal, and genital tissues, which are widely infected with one or more HHVs that are able to phosphorylate ACV.

RESULTS

ACV Suppresses HIV Replication in Human Lymphoid Tissue Coinfected Ex Vivo with HSV-2

In this study, we used an ex vivo system that preserves the cytoarchitecture of human tissues and supports replication of various viruses without exogenous stimulation (Glushakova et al., 1995).

Initially, we inoculated tissues with HIV-1_{LAI.04} and HSV-2 (strain G). Both viruses readily replicated in tissues. As expected, ACV at a concentration of 30 μ M suppressed HSV-2 replication in both HSV-2 singly infected and HIV-1 coinfecting tissues (by $97 \pm 1.8\%$ and $93 \pm 5.8\%$, respectively, $p = 3 \times 10^{-5}$, $p = 5 \times 10^{-4}$, $n = 4$). However, in these HSV-2-coinoculated tissues, ACV also suppressed HIV-1 replication by $62 \pm 4.2\%$ ($p = 2 \times 10^{-3}$, $n = 4$) (Figure 1A).

ACV Suppresses HIV Replication in Singly Infected Human Lymphoid Tissue Ex Vivo

Surprisingly, ACV was also found to suppress HIV-1 replication in tonsillar tissues from 38 donors that were not coinoculated with HSV-2 (Figures 1B–1D).

In HIV-1 singly infected tissues, the suppression of HIV-1 replication, measured as p24_{gag} accumulation in culture medium, was dose dependent with a 50% effective concentration (EC₅₀) of 3.1 μ M (95% confidence interval: 1.85–5.24) (Figure 1B). HIV-1 replication, evaluated by the measurement of HIV-1 proviral DNA in tissue blocks at day 12 postinfection, was reduced by 94.8% (interquartile range [IQR] 65.9%–99.8%) in blocks of tonsillar tissues treated with ACV at the concentration of 30 μ M compared with donor-matched untreated blocks. The median viral load was reduced by $\sim 1.3 \log_{10}$, from 3108 gag DNA copies per 10^4 cells in untreated tissues ($3.49 \log_{10}$; IQR 2.99–4.01) to 162 gag DNA copies per 10^4 cells in ACV-treated tissues ($2.27 \log_{10}$; IQR 0.09–3.07, $p < 10^{-4}$, $n = 27$) (Figure 1C). Consistently, ACV significantly decreased the amount of p24_{gag} released into the culture medium on average by $80 \pm 4\%$ ($p < 7 \times 10^{-7}$, $n = 38$) in human tonsillar tissues (Figure 1D). The average cumulative production of p24_{gag} in the culture medium of tonsillar tissues was 46.4 ± 8 ng/ml and 16.6 ± 4.6 ng/ml, respectively, in tissues untreated and treated with 30 μ M ACV.

To avoid the possible confounding effects of contaminating products in commercial ACV, we used ACV from three sources and found that all of them similarly suppressed HIV ($p \geq 0.45$, $n = 4$). Suppression of HIV-1 replication by ACV was not associated with T cell depletion, as evidenced from the similar numbers of total T cells (CD4⁺CD3⁺ and CD8⁺CD3⁺, $p \geq 0.5$, $n = 3$) and their subsets of activated (CD25⁺, HLA-DR⁺, or CD38⁺), naive (CD45RA⁺CD62L⁺), or nonnaive (CD45RA⁺CD62L⁺) ($p \geq 0.16$, $n = 3$) cells. Moreover, ACV antiviral activity was restricted to HSV-2 and HIV-1 since, as expected, ACV did not reduce vaccinia virus replication in tonsillar tissue ex vivo (data not shown).

ACV Suppresses Replication of CCR5- and CXCR4-Tropic HIV-1 Variants in Various Human Tissues

To test whether ACV suppressed HIV-1 replication in tissues other than tonsils, we used explants of tissues that also play an important role in HIV transmission and pathogenesis in vivo: lymph nodes and cervicovaginal and colorectal tissues. ACV suppressed HIV-1 replication, measured by p24_{gag} accumulation in culture medium, in lymph nodes by $70 \pm 13.9\%$ ($p = 2 \times 10^{-3}$, $n = 7$), in colorectal tissues by $72.6 \pm 9\%$ ($p = 2 \times 10^{-2}$, $n = 3$), and in cervical tissues by $60.1 \pm 3.5\%$ ($p = 7 \times 10^{-3}$, $n = 3$). ACV treatment of ex vivo human lymph nodes reduced the average cumulative production of p24_{gag} in culture medium from 67.3 ± 28.1 ng/ml to 15.4 ± 6.6 ng/ml, in ex vivo human colorectal tissues from 7.2 ± 6.8 ng/ml to 1.3 ± 1.2 ng/ml ($n = 3$), and in ex vivo human cervicovaginal tissues from 3.8 ± 1.7 ng/ml to 1.4 ± 0.5 ng/ml ($n = 3$) (Figure 2A).

Furthermore, we evaluated the efficiency of ACV in suppressing different HIV-1 variants. We inoculated ex vivo lymphoid tissues with CCR5- and CXCR4-tropic HIV-1 variants X4_{LAI.04}, R5_{SF162}, R5_{BaL}, and R5_{AD8}. As shown in Figure 2B, ACV suppressed replication of all of these isolates in tonsillar tissues with similar efficiency (by 88%, 95%, 89%, and 94%, respectively, as assessed from p24_{gag} production).

Coinfection with Different Endogenous Human Herpesviruses Is Associated with the Anti-HIV Effect of ACV

To test whether suppression of HIV by ACV is related to the antiherpetic activity of this drug, we measured the presence of various HHVs in the tonsillar tissues used in the present work (Figure 2C). Real-time PCR analysis revealed that all 27 tonsillar tissues that were tested for the presence of HHVs were negative for HSV-1, HSV-2, HHV-3 (VZV), and HHV-8 but were infected with HHV-4 (EBV), -5 (CMV), -6, and -7 in various combinations (Figure 2C). CMV was present in 15% of tissues; EBV, in 52%; HHV-7, in 89%; and HHV-6, in all but one tissue (96%).

Similar to what was observed in immunocompromised patients (Lusso and Gallo, 1994), HHV load was increased in tissues ex vivo. There was a 5-fold increase in the median HHV-6 load at day 12 in culture, from 22.4 DNA copies per 10^4 cells at the time of surgery (IQR 1.8–57.7) to 116.6 DNA copies per 10^4 cells (IQR 33.7–808, $p = 3 \times 10^{-4}$, $n = 26$). After ACV treatment, the median HHV-6 load at day 12 was reduced to 55.1 DNA copies per 10^4 cells (IQR 21.3–334.6, $p = 10^{-2}$, $n = 26$), demonstrating that ACV significantly suppressed HHV-6 replication. The sensitivity of HHV-6 to ACV was further evaluated in separate experiments in which ex vivo tonsillar tissues were inoculated with HHV-6B (PL-1 strain). In these experiments, ACV suppressed HHV-6B replication in a dose-dependent manner with an EC₅₀ of $\sim 27 \mu$ M. We found that ACV reduced the total production of HHV-6B in culture medium on average by $56.2 \pm 11.4\%$ ($52.2 \pm 12.4 \times 10^8$ DNA copies per ml versus $22.5 \pm 9.4 \times 10^8$ DNA copies per ml in control and ACV-treated tissues, respectively; $p = 3 \times 10^{-3}$, $n = 3$).

HHV-7, HHV-4, and HHV-5 median loads in tonsillar tissues were, respectively, 9.23 (IQR 0.51–56.81), 4.71 (IQR 0.06–448.3), and 0.1 (IQR 0.06–0.1) DNA copies per 10^4 cells at the time of surgery and 21.87 (IQR 0.9–219.7, $n = 24$), 31.16

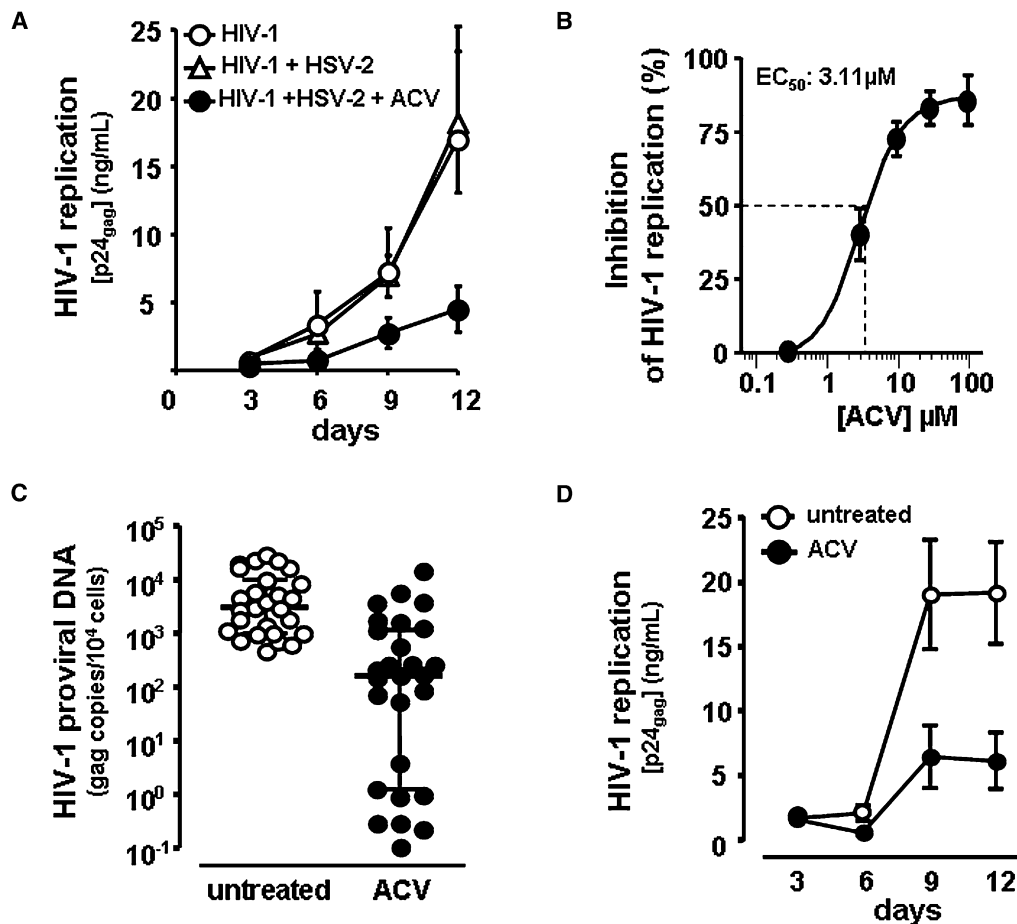


Figure 1. ACV Suppresses HIV Infection in Human Tonsillar Tissues

(A) Blocks of human tonsillar tissue were coinoculated ex vivo with HSV-2 strain G and X4_{LAI.04} and treated or not with ACV (30 μM). HIV-1 replication was monitored by measuring p24_{gag} accumulated in culture media over 3 day periods. Presented are means ± SEM of the results with tissues from 4 to 17 donors. For each donor, each data point represents pooled viral release from 27 tissue blocks. Note that ACV suppresses HIV-1 in HSV-2-coinfected tissues.

(B) HIV-1 replication was measured in tonsillar blocks infected with X4_{LAI.04} as in (A). ACV was added at the concentrations of 0.3, 3, 10, 30, and 100 μM, and its anti-HIV activity was evaluated from the suppression of viral replication compared with donor-matched HIV-infected tissues not treated with ACV. The 50% effective concentration (EC₅₀) was estimated by fitting the data to four-parameter logistic regression and was estimated to be 3.1 μM (95% confidence interval: 1.85–5.24). Presented are means ± SEM of the results with tissues from three to seven donors. Note that ACV suppresses HIV-1 replication in the tonsillar tissues in a dose-dependent manner.

(C) HIV-1 proviral DNA load in tissue blocks at day 12 postinfection was measured by real-time PCR. Presented are medians and interquartile ranges of the results (n = 27). Note that ACV efficiently reduces HIV-1 proviral DNA load in blocks of tonsillar tissue.

(D) HIV-1 replication was monitored as in (A). Presented are means ± SEM of the results (n = 38). Note that ACV efficiently suppresses HIV-1 replication in the tonsillar tissues tested.

(IQR 3.63–260.9, n = 14), and 388 (IQR 175.9–2416, n = 4) copies per 10⁴ cells after 12 days of culture.

In all tissues infected with different combinations of HHVs, ACV suppressed HIV-1 replication with a similar efficiency (Figure 2C). HHV-6 was the only HHV that was present in all combinations. Moreover, it seems that the suppression of HIV-1 by ACV is related to the amount of HHV-6. In tissue blocks in which ACV inhibited HIV-1 replication by more than 50%, the median HHV-6 load on day 12 in culture was significantly higher than in the tissues in which ACV suppressed HIV-1 replication by less than 50%: 131.1 DNA copies per 10⁴ cells (IQR 36.1–1154, n = 23) versus 21.6 DNA copies per 10⁴ cells (IQR 0.6–77.4, n = 4; p = 4 × 10^{−2}), respectively. Furthermore, we documented a correlation between the ACV-mediated suppression

of HIV-1 replication, as measured by p24_{gag} release, and the level of HHV-6 in tissues where the HIV-1 inhibition was suboptimal (between 0 and 99%; r = 0.43, p = 0.03, n = 25).

Although several HHVs, such as HHV-6, are ubiquitous and transmitted early in childhood, we identified a tissue in which there was no detectable HHV. In agreement with our hypothesis, in this tissue, ACV suppression of HIV-1 (at the concentration of 30 μM) was negligible (16%) (Figure 2C).

Coinfection with HHVs Is Necessary and Sufficient for the Anti-HIV Effect of ACV

To prove this claim, we performed experiments with MT4 cells, an HHV-uninfected T cell line that efficiently supports the replication of HIV-1_{LAI.04}. We found that ACV did not suppress

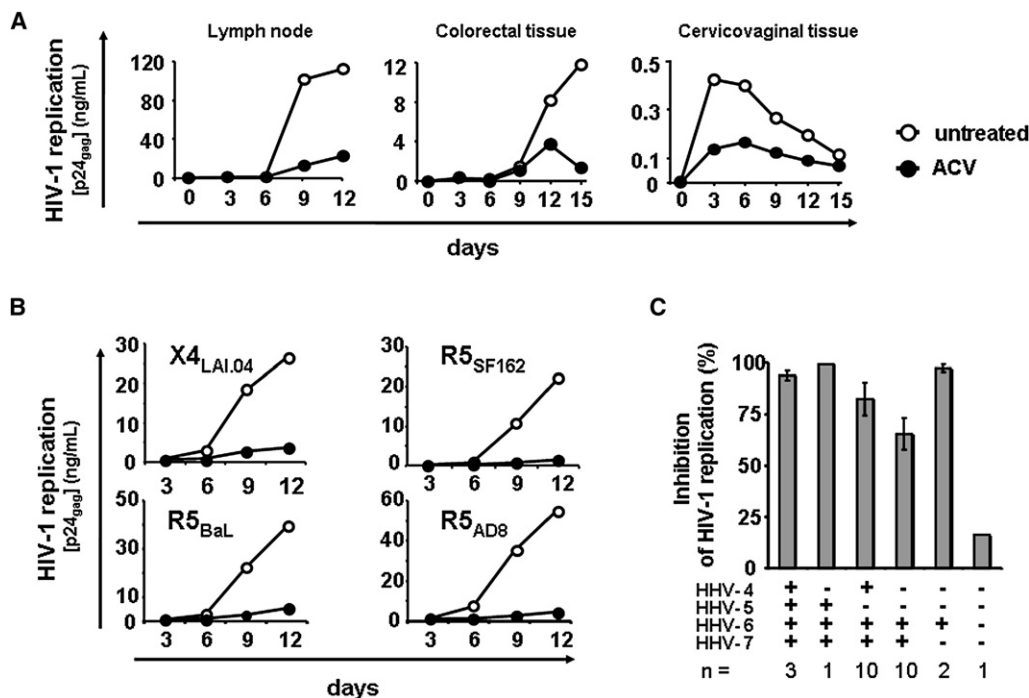


Figure 2. ACV Suppresses Replication of Different HIV-1 Variants in Human Tissues Coinfected with Various HHVs

(A) HIV-1 replication in blocks of human lymph nodes and colorectal and cervicovaginal tissues was monitored as in Figure 1A. For each type of tissue, the graph represents a typical result of three to seven experiments performed with tissues from different donors. Note that ACV efficiently suppresses replication of HIV-1 in lymph nodes and colorectal and cervicovaginal tissues.

(B) Examples of ACV suppression of different HIV-1 variants (X4_{LAI.04}, R5_{BaL}, R5_{SF162}, and R5_{AD8}). Each data point represents pooled viral release from 27 tissue tonsillar blocks. Note that ACV efficiently suppresses replication of all four HIV-1 variants.

(C) Presence of HHV-1, -2, -3, -4, -5, -6, -7, and -8 by real-time PCR in blocks of tonsillar tissue. All tissues were negative for HHV-1, -2, -3, and -8. Presented are means \pm SEM of the results with tonsils from n donors. Note that there are no significant differences in the level of ACV suppression of HIV-1 replication in tissues infected with various HHVs.

HIV-1_{LAI.04} ($EC_{50} > 250 \mu M$) in these cultures, in agreement with earlier observations (Barral et al., 2003). To test whether HHV infection is sufficient for the anti HIV-1 effect of ACV, we added various amounts of HHV-6B-infected MT4 cells to HHV-free MT4 cultures infected with HIV-1_{LAI.04}. As expected, ACV suppressed HHV-6. After 3 days of culture, the fraction of HHV-6B-infected cells was reduced in a dose-dependent manner, as measured by flow cytometry (Figure 3A). Importantly, in these cultures, ACV suppressed HIV replication as well, as evaluated by the number of p24_{gag}⁺ T cells and p24_{gag} release into the culture medium (EC_{50} of $\sim 50 \mu M$) (Figures 3A and 3B). In these experiments, 9% of HHV-6B-infected cells were sufficient to suppress HIV-1_{LAI.04} replication by $\sim 70\%$ (Figure 3A). We found that, in agreement with earlier publications (De Clercq et al., 2001), the concentration that corresponds to the EC_{50} in these experiments did not affect cell viability (50% cytotoxic concentration $> 250 \mu M$).

To further prove that ACV phosphorylation is required for HIV-1 inhibition, we synthesized the monophosphorylated ACV prodrug acyclovir-(1-naphthyl [methoxy-L-alaninyl]) phosphoramidate (Cf2649) (Figure 3C). This compound bypasses the requirement of HHVs for the activation of ACV since it is already monophosphorylated. Indeed, when applied to (HHV-free) MT-4 cells, Cf2649, in contrast to nonphosphorylated ACV ($EC_{50} > 250 \mu M$), suppressed HIV-1 replication with an EC_{50} of $\sim 3 \mu M$

(Figures 3D and 3E). Similar results were obtained with another HHV-free T cell line (CEM) (data not shown).

In conclusion, we demonstrated that neither in HHV-free tissue nor in HHV-free cell lines does ACV suppress HIV-1 infection. Reconstitution of the cell line system with HHV-6-infected cells or bypassing the HHV-kinases requirement by applying an ACV monophosphate derivative makes HHV-free systems susceptible to HIV suppression by ACV.

ACV-TP Inhibits HIV-1 RT

Since we demonstrated that the anti-HIV-1 activity of ACV requires HHV-mediated activation and that phosphorylated derivatives of ACV are produced in HHV-infected tissues (see Supplemental Data and Figure S1 available online), we hypothesized that ACV-TP, into which ACV is ultimately converted (Elion, 1983), interferes with the activity of the HIV-1 RT and directly suppresses HIV-1 replication.

We tested whether ACV-TP suppresses RT by measuring the polymerizing activity of RT from lysed HIV-1 using an exogenously added HIV-1 template. We observed a dose-dependent inhibition of HIV-1 RT activity by ACV-TP, while we noted no suppression of HIV-1 RT activity by ACV itself, even at high concentrations (Figure 4A).

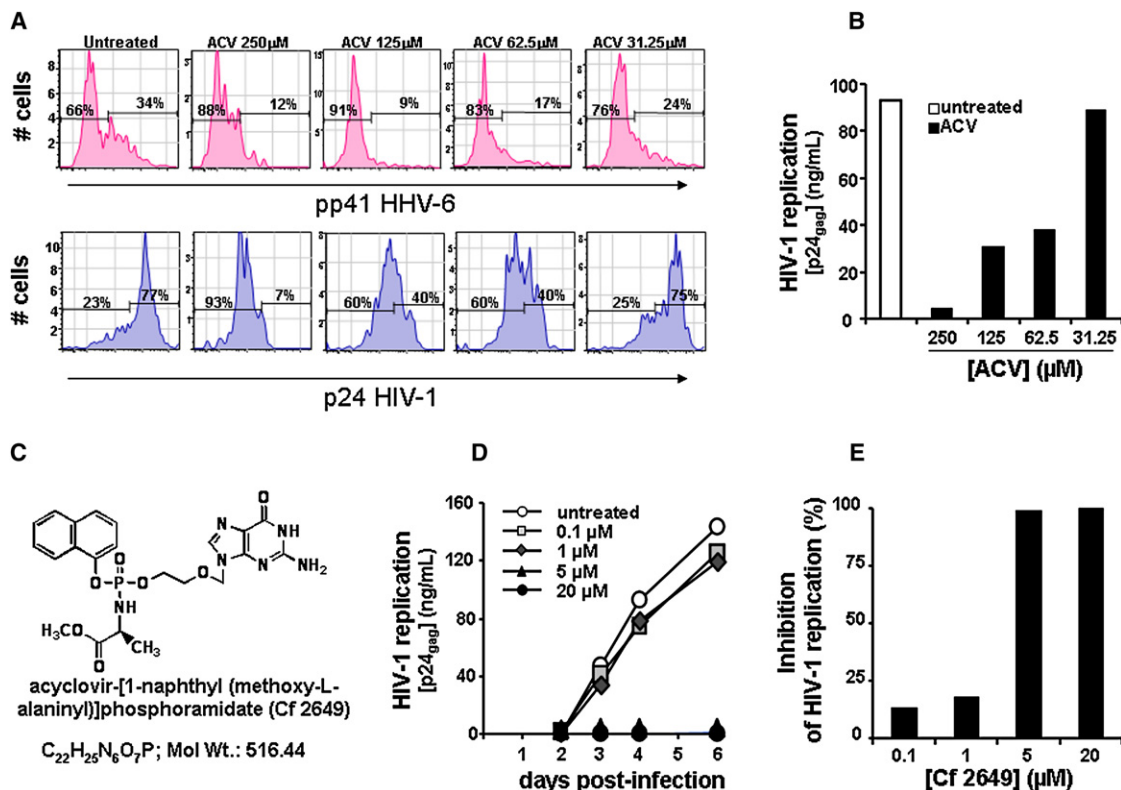


Figure 3. ACV Suppresses HIV-1 in HHV-Free MT-4 Cell Cultures Either in the Presence of HHV-6-Infected Cells or as Monophosphorylated Prodrug

(A) HIV-1_{LAI.04}-infected MT4 cells were cocultured with HHV-6B-infected MT4 cells in a ratio of 10:1. Presented are the distributions of HHV-6B (upper panel) and HIV-1_{LAI.04}-infected cells (lower panel), as measured by flow cytometry at day 3 post-HIV-1 infection, in cultures untreated or treated with various concentrations of ACV. Note that the fraction of both HHV-6B- and HIV-1-infected cells is reduced in a dose-dependent manner by ACV treatment.

(B) HIV-1_{LAI.04}-infected MT4 cells were cocultured with HHV-6B-infected MT4 cells. HIV-1 replication was monitored as in Figure 1A. Note that the replication of HIV-1 in HHV-6B/HIV-1-infected cocultures is suppressed by ACV treatment in a dose-dependent manner.

(C) The compound acyclovir-[1-naphthyl (methoxy-L-alaninyl)] phosphoramidate (Cf2649) has been synthesized as described in the Supplemental Data.

(D) HIV-1_{LAI.04}-infected HHV-free MT4 cells were treated with various concentrations of the ACV monophosphorylated prodrug Cf2649. Presented are the kinetics of HIV-1_{LAI.04} replication, monitored by measuring p24_{gag} accumulated in culture media at days 2, 3, 4, and 6 postinfection. Presented data are representative of two experiments. Note that compound Cf2649 suppresses the replication of HIV-1_{LAI.04} in a dose-dependent manner.

(E) HIV-1_{LAI.04}-infected MT4 cells not infected with any HHVs were treated with various concentrations of the compound Cf2649. The cumulative release of p24_{gag} into culture media over 6 days of culture treated with various concentrations of the compound Cf2649 is presented as a fraction of the p24_{gag} production in the untreated cultures. Presented data are representative of two independent experiments. Note that compound Cf2649 suppresses the replication of HIV-1_{LAI.04} in a dose-dependent manner.

Since ACV-TP is a guanosine-5'-triphosphate analog, we investigated whether it acts as a nucleotide RT inhibitor by testing whether dGTP prevents ACV-TP inhibition of RT. We found that, at ACV-TP concentrations of 3.38 μ M or 33.8 μ M, HIV-1 RT inhibition was inversely dependent on the dGTP concentrations. At the highest concentration of ACV-TP tested, no competition with dGTP was observed (Figure 4B). Since ACV-TP lacks an additional hydroxyl group (present in dGTP and essential for DNA chain polymerization), we further hypothesized that the above-described suppression of HIV-1 RT by ACV-TP is similar to its suppression of HSV DNA polymerase, namely by incorporation into the nascent HIV DNA resulting in its chain termination (Reardon and Spector, 1989).

To prove this hypothesis, we used a gel-based assay (Marchand and Gotte, 2003; Marchand et al., 2007) and found that HIV-

1 RT incorporated ACV-TP into the DNA primer as efficiently as the natural substrate dGTP. The efficiency of single-nucleotide incorporation events (k_{cat}/K_m) under steady-state conditions for dGTP and ACV-TP was 14.7 μ M⁻¹min⁻¹ and 14.0 μ M⁻¹min⁻¹, respectively (Table S1). Furthermore, ACV-TP incorporation resulted in complete DNA chain termination, as shown in Figures 5A and 5B. A primer that contained the natural dGMP at its 3' end was successfully extended in the presence of dTTP, which is the substrate for the following three consecutive template positions (Figure 5B, left panel). In contrast, DNA synthesis was effectively blocked when the primer was terminated with ACV-MP. Even the relatively high concentration of 50 μ M of dTTP did not permit its incorporation (Figure 5B, right panel). These data suggest that the mechanism of action of ACV is similar to the currently approved anti-HIV nucleoside reverse transcriptase inhibitors (NRTIs).

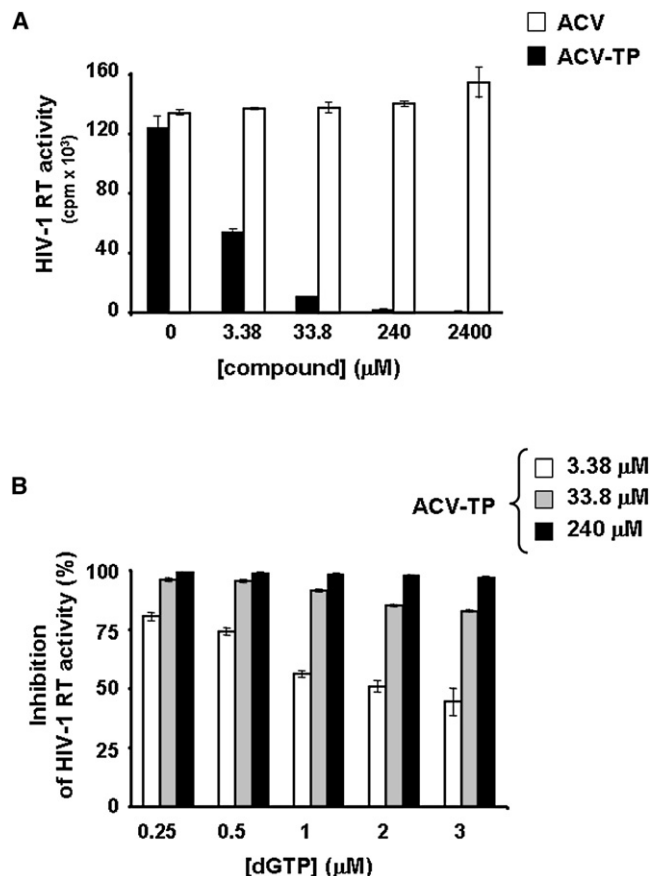


Figure 4. ACV-TP Inhibits HIV-1 RT in Exogenous Template Reverse Transcriptase Assays

(A) Exogenous template reverse transcriptase assays were performed in the presence of various concentrations of ACV or ACV-TP as described in the [Experimental Procedures](#). Presented are means \pm SEM of the results of two experiments performed in duplicates. Note that ACV-TP inhibits HIV-1 RT in a dose-dependent manner.

(B) The dependence of the RT inhibition by ACV-TP on the concentration of dGTP was evaluated using an exogenous template reverse transcriptase assay. dGTP was used at the indicated concentrations. The reactions were performed in the presence of the indicated concentrations of ACV-TP. Presented are means \pm SEM of the results of two experiments performed in duplicate. Note that inhibition of HIV-1 RT activity by ACV-TP is inversely dependent on the concentration of dGTP.

DNA chain termination by NRTIs is not irreversible but can be subjected to phosphorolytic excision of the incorporated drug by HIV RT ([Meyer et al., 1998](#)). Binding of the next complementary nucleotide following the DNA chain terminator can lead to the formation of a dead-end complex (DEC). In this complex, HIV RT is trapped in a conformation that blocks the excision reaction. Thus, to test whether ACV-MP-terminated DNA is accompanied with the formation of a dead-end complex, we evaluated the inhibition of ACV-MP phosphorolytic excision from the 3' end of the primer by HIV-1 RT. The excision of a DNA chain terminator requires the presence of pyrophosphate (PPi) or pyrophosphate donor molecules (such as ATP). We found that HIV-1 RT is capable of excising the incorporated ACV-MP in the presence of physiologically relevant concentrations of ATP (data not shown)

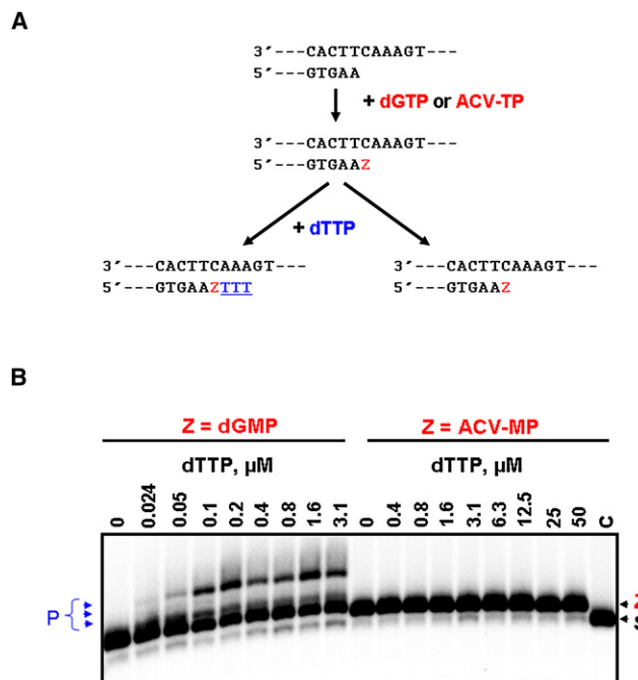


Figure 5. Chain Termination with ACV-MP

(A) The reaction scheme shows the relevant region of the primer and template and two possible outcomes of the primer elongation. Elongation of the primer in the presence of dGTP or ACV-TP is indicated by the "Z" in red, which refers to the incorporated monophosphate dGMP or ACV-MP, respectively. Incorporation events following position Z with dTTP are in blue and underlined. (B) The primer (S, lane C) was initially elongated to incorporate dGMP or ACV-MP, respectively, at the 3' end referred to as Z. Incubation with increasing concentrations of the next nucleotide dTTP resulted in three nucleotide incorporation events with the dGMP-terminated primer, which are labeled as products (P). Note that the ACV-MP-terminated primer is not extended, which shows that the inhibitor acts as a chain terminator.

as demonstrated for certain NRTIs. However, to analyze whether the ACV-terminated primer permits formation of a dead-end complex, the ATP-dependent excision reaction was assayed in the presence of increasing concentrations of the next complementary nucleotide. We found that the next complementary nucleotide caused 50% inhibition of ACV-MP excision (IC_{50}) at a concentration of ~ 26 μ M ([Figures 6A](#) and [6B](#)). These data strongly suggest the formation of a dead-end complex in ACV-MP-incorporated templates. The higher IC_{50} (26 μ M versus 3.1 μ M) in comparison to reactions conducted with the control DNA chain terminator ddGMP was probably due to the acyclic nature of ACV-MP. In general, the excision reaction was inefficient; only 10%–15% of the terminated primer strands were rescued for continuation of DNA synthesis at low concentrations of the next complementary nucleotide.

To provide additional direct evidence of dead-end complex formation, we employed site-specific footprinting that allowed us to determine the position of HIV-1 RT on its primer/template at single-nucleotide resolution ([Marchand and Gotte, 2003](#); [Marchand et al., 2007](#)). Nucleotide binding and formation of a dead-end complex can only occur in the posttranslocated conformation in which the nucleotide-binding site of HIV-1 RT has

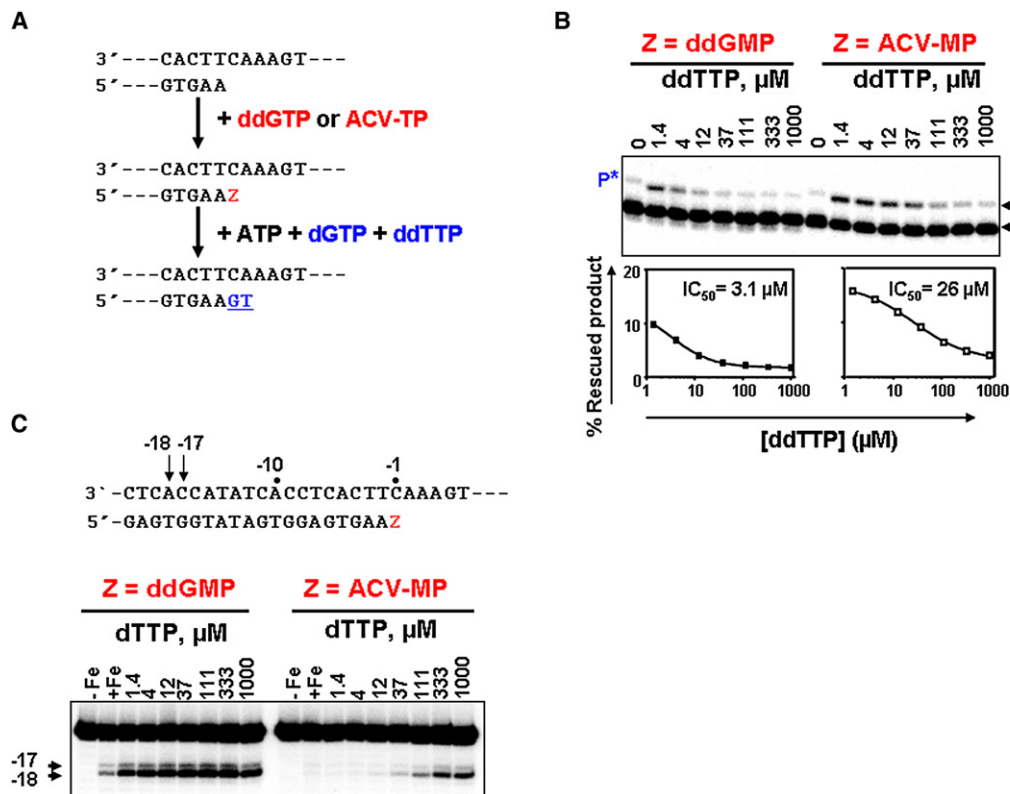


Figure 6. Inhibition of ATP-Dependent Excision in the Presence of the Next Nucleotide Substrate

ATP-dependent excision was monitored in a combined excision/rescue assay as previously described in the [Supplemental Experimental Procedures](#).

(A) The reaction scheme shows the relevant region of the primer and template. Extension of the primer in the presence of ddGTP or ACV-TP is indicated by the “Z” in red. The excision of a DNA chain terminator requires the presence of ATP as pyrophosphate donor. The 3'-ultimate nucleotide (Z) was excised with ATP, and the simultaneous presence of dGTP and ddTTP (in blue) allowed the rescue of DNA synthesis.

(B) The combined excision/rescue reaction was studied with ddGMP and ACV-MP-terminated primers. Z refers to the terminated primer and P to the rescued product. The asterisk indicates dGMP misincorporation in the absence of the correct ddTTP substrate. Quantification of excision/rescue reactions for ddGMP and ACV-MP are plotted. The concentration of the next complementary nucleotide required to inhibit 50% of the reaction is calculated on the basis of these two curves. Note that ACV-MP-terminated primer excision/rescue reaction is inhibited due to dead-end complex formation.

(C) Incorporation of ACV-TP produces a dead-end complex with HIV-1 RT. Presented is site-specific footprinting of HIV-1 RT with ddGMP- and ACV-MP-terminated primers in the presence of increasing concentrations of the next complementary nucleotide. Lanes -Fe and +Fe show control reactions in the absence and presence of divalent Fe^{2+} ions that cause site-specific cleavage on the labeled template. The arrows and the sequence underneath the gel show the position of the oxidative cleavage on the template strand at positions -17 and -18, which are indicative for post- and pretranslocated complexes, respectively. Note that, for ACV-MP-terminated primer, no cleavage occurs at low next complementary nucleotide concentrations, indicating that the complex between RT and terminated primer is fragile, whereas, at higher concentrations of the next complementary nucleotide, RT is blocked in a dead-end complex.

been cleared. In this conformation, the RT has moved a single nucleotide further downstream relative to the pretranslocated state. Our footprints are based on oxidative cleavage on the template strand at positions -17 and -18, which are indicative of post and pretranslocated complexes, respectively. We compared footprints of complexes with ddGMP- and ACV-MP-terminated primers in the presence of increasing concentrations of the next complementary nucleotide (Figure 6C). Low concentrations of the next complementary nucleotide are sufficient to stabilize the posttranslocated complex when the primer was terminated with ddGMP, proving the formation of a dead-end complex. A similar pattern is seen with ACV-MP, although the concentration of the next nucleotide required to stabilize the complex is higher. Oxidative cleavage in case of ACV-MP did not occur in the absence of or at low concentrations of the next complementary nucleotide. These findings suggest that, at the low next comple-

mentary nucleotide concentration, the complex between RT and terminated primer formed at the site of the ACV-MP termination is relatively fragile—perhaps prone to dissociation. In contrast, at high concentration of the next complementary nucleotide, RT can be trapped in a dead-end complex. The dissociation constant for ACV-terminated primers in the presence of a high concentration of the next nucleotide (111 μM) is increased ~ 3 -fold (Figure S2).

Thus, these data explain why the excision of ACV-MP is generally inefficient at both low and high concentrations of the next complementary nucleotide. At low concentrations, the complex between RT and ACV-MP is relatively unstable, while, at higher concentrations, a DEC is formed.

To prove that ACV targets HIV-1 RT in human tissues as in cell-free assays, we tested two pairs of HIV isogenic strains that are resistant to some of the currently approved NRTIs. The isolate

AZT.4x containing the mutations at D67N, K70R, T215Y, and K219Q express complete resistance to AZT and resulted to be as sensitive to ACV in infected tissues as its parental HIV-1 variant (Figure S3A). However, an HIV-1 isolate carrying the M184V mutation (that confers resistance to Lamivudine) was less sensitive to ACV than its parental strain (Figure S3B). The EC_{50} for the M184V isolate was approximately four times higher than that for the parental HIV-1 isolate. Thus, the evidence that a specific isolated mutation of one amino acid in RT can reduce the sensitivity of an HIV-1 isolate to ACV further proves that HIV-1 RT is targeted and suppressed by ACV in human tissues, in agreement with the data on ACV suppression of HIV-1 RT in cell-free systems.

DISCUSSION

Growing epidemics of HIV-1 infection, especially in countries with limited resources, and the emergence of drug-resistant viruses make it necessary to find ever new safe, efficient, and inexpensive strategies against this virus. Strategies as effective and safe as those developed against several other human pathogens such as HSV, which can be efficiently treated with ACV, have yet to be developed against HIV. The antitherpetic specificity of ACV is primarily based on the unique ability of HHV-encoded kinases to phosphorylate ACV to its monophosphate derivative, which is subsequently converted into the antivirally active ACV-TP (Reardon and Spector, 1989). Consistent with its highly restricted antitherpetic activity, ACV is not currently used as a direct HIV-1 inhibitor.

Our findings demonstrate (1) the direct inhibitory effect of ACV-TP, but not of ACV itself, on isolated HIV-1 RT in a cell-free system, (2) the suppression of HIV-1 replication by ACV in human tissues and in T cell lines if and only if they carry HHV that phosphorylate ACV, and (3) the ability of a phosphorylated ACV prodrug to bypass the requirement of HHV to suppress HIV. Thus, we provide definitive experimental evidence of inhibition of HIV-1 RT activity by phosphorylated ACV and demonstrate that ACV phosphorylation occurring in HHV-infected human tissues transforms this widely used antitherpetic drug into an HIV-1 inhibitor.

The direct suppression of HIV-1 RT activity by ACV-TP was demonstrated in two different cell-free assays, one using recombinant HIV-1 RT and the other using RT extracted from lysed HIV-1 virions. We showed that ACV-TP is incorporated into the nascent viral DNA chain with a level of efficiency similar to that of its natural equivalent dGTP. Incorporation of ACV-TP results in the termination of reverse transcription, while the excision of the incorporated ACV-MP from the DNA chain is partially inhibited because of the fragility of the complex between HIV-1 RT and the terminated DNA chain or because of the formation of a dead-end complex. These properties of ACV resemble those of certain approved anti-HIV NRTIs (Marchand and Gotte, 2003; Meyer et al., 1999), and we showed here that ACV-TP, a nonphosphonate acyclic nucleoside analog, inhibits HIV-1 RT.

To further confirm that, in HIV-1-infected cells, ACV-triggered HIV-1 suppression is mediated by RT, we identified a pair of isogenic HIV-1 strains differing only by one amino acid (M184V) in RT but having different sensitivities to ACV in ex vivo human tissues. Future studies on selection of ACV-resistant HIV-1 isolates

and testing of various multidrug-resistant HIV-1 isolates for ACV sensitivity will reveal the exact set of RT mutations associated with reduced sensitivity to ACV and the rate of their evolution. Identification of ACV-resistant mutants does not, in general, exclude the use of ACV against HIV-1 variants resistant to other NRTIs; for instance, ACV efficiently suppresses the replication of AZT-resistant HIV-1.

As demonstrated in a cell-free system, to inhibit HIV-1 RT, ACV has to be converted into ACV-TP. In human tissues, such conversion requires HHV infection. Accordingly, we found that, in the presence of HHVs that are capable of phosphorylating ACV, HIV-1 was inhibited in ACV-treated tonsils, lymph nodes, and cervico-vaginal and colorectal tissues, where the critical events of HIV-1 pathogenesis and transmission occur in vivo.

Various HHVs, including the ubiquitous HHV-6 detected in all but one tissue ex vivo, may mediate HIV-1 suppression by ACV. The level of HHV-6 replication may be essential. In tonsillar tissues, in which ACV inhibited HIV replication by more than 50%, the median HHV-6 load was significantly higher than in the tissues in which ACV suppressed HIV replication by less than 50%. Moreover, in tissues where the HIV-1 inhibition was suboptimal, there was a correlation between the ACV-mediated suppression of HIV-1 replication and the level of HHV-6. These results demonstrate again the critical role of HHVs in ACV-mediated suppression of HIV-1 and indicate the importance of HHV-6 in mediating HIV-1 suppression by ACV in our ex vivo tissue system.

HHV-6 and HHV-7 are ubiquitous viruses, and, therefore, the probability of finding an HHV-free tissue is very low. Nevertheless, by testing tissues from multiple donors, we identified one tonsillar tissue that was not infected by any HHV. ACV did not inhibit HIV-1 replication in this tissue.

However, to further demonstrate that HHVs are necessary and sufficient for the anti-HIV effect of ACV, we used the HHV-uninfected MT4 cell line. Consistent with the proposed mechanism based on HHV-mediated activation, ACV did not inhibit HIV replication in this HHV-free cell line. However, when HHV-6-infected cells were added to the HIV-1-infected cultures, ACV became an HIV-1 suppressor. This effect was dependent on both the concentration of ACV and the fraction of HHV-6-infected cells. Apparently, in HHV reconstituted MT4 cultures, ACV that is phosphorylated in HHV-6-infected cells is transferred to HIV-1-infected cells, since the majority of these cells were not coinfecting with HHV-6. These results are in full agreement with the published data on the transfer of phosphorylated ACV between cells (Burrows et al., 2002; Degreve et al., 1999). In tissues, transfer of phosphorylated ACV between cells is facilitated by specialized contacts (Nicholas et al., 2003). These and probably other factors (e.g., rapid proliferation and large endogenous dNTP pools, as well as a high level of HIV-1 replication) may contribute to a lower sensitivity of HIV-infected cell lines to ACV compared with integral tissues.

To further demonstrate the necessity of ACV activation for HIV suppression, we synthesized an already monophosphorylated (activated) ACV in which the phosphate is masked by lipophilic groups. In contrast to nonphosphorylated ACV, this prodrug suppressed HIV-1 in HHV-free cultures of MT4 or CEM cells. Although enzymatic reactions mediating ACV prodrug conversion into its active form have been described (Congiatu et al., 2007),

the entire process of this conversion remains to be elucidated. Nevertheless, our results provide strong evidence that, upon phosphorylation, ACV suppresses HIV-1 replication in cells. Importantly, these experiments also demonstrate the feasibility of designing a new class of anti-HIV compounds. However, unlike ACV (with a proven safety record and exhaustively studied pharmacokinetics), ACV prodrug effects in various systems have to be evaluated in order to form conclusions on their potential clinical use.

In summary, the following mechanism seems to be responsible for ACV suppression of HIV-1 in human tissues *ex vivo*, the majority of which carry one or several HHVs, including HHV-6: ACV is monophosphorylated by herpesviral enzymes in HHV-infected cells and then further converted to ACV-TP, which suppresses HIV by inhibiting HIV-1 RT, similarly to other NRTIs.

Our results suggest that ACV may be therapeutically beneficial for various HIV-1-infected patients, since the majority of humans are already infected with HHV-6, often together with other HHVs that activate ACV at least during reactivation. In particular, in immunocompromised patients for whom HHV replication is frequent, those HHVs that are not completely suppressed by ACV (e.g., HHV-6) can continuously generate phosphorylated ACV derivatives. The incomplete inhibition of HHV-6 by ACV confirmed in our experiments is consistent with the much higher K_i of ACV-TP for HHV-6 DNA polymerase (UL69) compared with that of HSV-2 or HSV-1 DNA polymerase (Bapat *et al.*, 1989).

However, clinical trials are needed to test whether replication of HHVs—in particular of HHV-6, which is typically maintained in various organs, including the intestines and the vagina, even in immunocompetent individuals (De Bolle *et al.*, 2005)—would be sufficient to suppress HIV-1 in ACV-treated individuals. Also, clinical trials should reveal whether the range of ACV concentrations used in our study to suppress HIV-1 is clinically relevant. Although ACV penetration efficiency and drug clearance were unknown for *ex vivo* tissues, the calculated EC_{50} of 3.1 μ M was in the range of what was reported *in vivo*: a dose of 1 g of oral valacyclovir per day results in a plasma peak concentration of 29.5 μ M, a minimum concentration in serum of 3 μ M, and a plasma concentration time curve of 89 μ M/h ACV (Lycke *et al.*, 2003; Soul-Lawton *et al.*, 1995). Moreover, the therapeutic dose of the orally administered ACV prodrug valacyclovir, depending on clinical indications, can be increased to as much as 3 g per day.

Recent clinical trials performed so far are in agreement with our *ex vivo* results and demonstrated that ACV is efficient in suppressing HIV in HSV-2-coinfected individuals (Baeten *et al.*, 2007; Delany *et al.*, 2007; Dunne *et al.*, 2008; Nagot *et al.*, 2007; Zuckerman *et al.*, 2007). Valacyclovir treatment, at the dose of 1 g per day, reduced the HIV-1 plasma load in these individuals by 50%–70%, an effect comparable to that reported here for human tissues infected with other HHVs. This HIV-1 viral load reduction was clinically beneficial (Corey, 2007) and similar to that reported for AZT or stavudine monotherapy (~70%) (Delta Coordinating Committee and Delta Virology Committee, 1999; Katzenstein *et al.*, 2000; Rey *et al.*, 1998). In contrast to the established ACV activity in reducing HIV load in HSV-2 coinfected patients, recent trials failed to demonstrate that HSV-2 suppressive therapy prevents acquisition of HIV-1 (Celum *et al.*, 2008; Cohen, 2007; Lisco and Vanpouille, 2008; Watson-Jones *et al.*,

2008). However, none of the approved NRTIs widely used for therapy was yet developed into an efficient preventive drug when used alone.

Our results provide new insights into the effect of ACV in HIV-1-infected patients. In all previous trials, the effect of ACV on HIV-1 was considered to be indirect and due to the suppression of HSV-2-mediated inflammation. Here, we demonstrate that ACV directly suppresses HIV-1 RT in HHV-coinfected tissue. This effect depends on the levels of HHVs, which were not evaluated in these clinical trials or in previous *in vitro* studies (Resnick *et al.*, 1986). Obviously, the results obtained in tissues *ex vivo* should be extrapolated to the *in vivo* situation with caution. However, the reliance on lymphotropic HHVs to create the active HIV suppressor at the site of HIV replication may, in principle, solve the critical pharmacological problem of drug delivery.

In conclusion, our data on HIV-infected tissues coinfected with various HHVs suggest that ACV may be used to decrease the HIV load in both the peripheral blood and the genital compartments of patients infected with one or several HHVs, including the highly prevalent HHV-6 (Campadelli-Fiume *et al.*, 1999). Although the magnitude of HIV-1 suppression by ACV as well as by the currently approved NRTIs is too low to be used in monotherapy, it is sufficient to be an important part of drug cocktails.

In general, the combination of ACV with an endogenous HHV infection to suppress HIV may constitute a new principle of anti-HIV therapy—a “binary weapon” in which one inert component is converted by another endogenous component into an active therapeutic compound. In the case of ACV, its exceptionally low toxicity and the low cost of ACV and related drugs that have been safely used in humans for more than 30 years, as well as their existing formulations as pills and creams, make them potentially applicable for HIV treatment, possibly in combination with other drugs. New targeted clinical trials will test whether ACV and its derivatives can be used for this new purpose in line with a popular trend to identify new uses for old drugs (Chong and Sullivan, 2007).

EXPERIMENTAL PROCEDURES

Tissue and Cell Culture

Tonsillar tissues from routine surgery were obtained from the Children's National Medical Center (Washington, DC). Lymph nodes and colorectal and cervicovaginal tissues were obtained either from routine surgery or from cadavers through the National Disease Research Interchange (Philadelphia, PA). All tissues were obtained according to IRB-approved protocols. Tissues were dissected into 2 mm³ blocks and cultured as described earlier (Fletcher *et al.*, 2006; Glushakova *et al.*, 1995; Grivel *et al.*, 2007). MT4 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium with 10% heat-inactivated FCS.

Viral Infections

Tissue inoculation with HIV-1 and HHV-6B was performed as described earlier (Grivel *et al.*, 2007). For further details, see the [Supplemental Experimental Procedures](#).

ACV and ACV Monophosphorylated Prodrug Treatment

ACV pharmaceutical formulations for intravenous infusion (Bedford Laboratories, Bedford, OH; NDC 55390-612-10) were diluted in distilled water and used at the indicated concentrations. A different pharmaceutical formulation for intravenous infusion (American Pharmaceutical Partners, Schaumburg, IL; NDC 63323-325-10) and a commercial preparation of ACV (Sigma-Aldrich, St. Louis, MO) were tested for HIV-1 suppression in matched tonsillar tissues

from four donors, with similar results. Synthesis of the monophosphorylated ACV prodrug-acyclovir-(1-naphthyl [methoxy-L-alaninyl]) phosphoramidate (Cf2649) is described in the [Supplemental Experimental Procedures](#). Compounds were added to the culture medium 12 hr prior to HIV-1 infection and again at each culture medium change.

Real-Time PCR

The HHV load and the HIV-1 proviral load in tissues were determined by measurement of the number of viral DNA copies. DNA from two blocks of tissue was extracted with the QIAamp kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. For further details on real-time PCR TaqMan assay, primers, and probes sequences, see the [Supplemental Experimental Procedures](#).

Exogenous Template RT Assay

Exogenous template HIV-1 RT assays were performed as described previously (Gorelick et al., 1990), with modifications specified in the [Supplemental Experimental Procedures](#).

Steady-State Kinetics, ATP-Dependent Excision, and Site-Specific Footprinting

These assays were performed as described previously (Gotte et al., 1998; Marchand and Gotte, 2003; Marchand et al., 2007) with modifications specified in the [Supplemental Experimental Procedures](#).

Statistical Analysis

Each datum point is the result of analysis of sets of 9 to 27 tissue blocks derived from each of *n* donors, where *n* is indicated in the text. Since the absolute level of HIV-1 replication varied from donor to donor, for every experiment, we compared these levels using data from matched control blocks as the basis of normalization. This allowed us to pool results obtained from tissues from different donors. We analyzed these data using a two-tailed paired Student's *t* test. Because the distribution of the numbers of HHV-6 and HIV-1 (proviral) DNA equivalents failed the Kolmogorov-Smirnov normality test, we used distribution-free nonparametric methods (Wilcoxon Match-Pairs, Signed-Ranks Test, and Mann-Whitney U test) to evaluate the significance of the differences between various experimental groups. However, when these data were log₁₀ transformed, the normality was achieved and we applied parametric methods (paired or unpaired Student's *t* test). The statistical significance of differences between data from various experimental groups evaluated after transformation was similar to that evaluated with nonparametric methods applied to non-transformed results. Depending on the type of statistical analysis, the pooled data are presented either as means \pm standard error of the mean (SEM) or as median and interquartile range (IQR). All of the hypothesis tests were two-tailed, and a *p* value of ≤ 0.05 defined statistical significance.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at <http://www.cellhostandmicrobe.com/cgi/content/full/4/3/260/DC1/>.

ACKNOWLEDGMENTS

We thank Dr. M. Santi and the entire staff of the Department of Pathology of Children's National Medical Center for their generous assistance in obtaining human tonsillar tissues.

This research was supported, in part, by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, and by federal funds from the National Cancer Institute, NIH, under contract NO1-CO-12400. R.F.S. is supported, in part, by NIH grants 5P30-AI-50409 (CFAR), 5R37-AI-041980, and the Department of Veterans Affairs. J.B. is supported by the Geconcerteerde Onderzoeksacties (GOA No. 05/19). M.G. is the recipient of a national career award and a research grant from the Canadian Institutes of Health Research. L.M. dedicates this paper to the 80th birthday of his mentor, Professor Ju. M. Vasiliev.

Received: April 28, 2008

Revised: June 15, 2008

Accepted: July 16, 2008

Published: September 10, 2008

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